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Enzyme-Linked Immunosorbent Assay Using Recombinant Antigens for Serodiagnosis of Japanese Encephalitis

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Recombinant Japanese encephalitis (JE) virus proteins were evaluated as antigens for serodiagnosis of JE using an enzyme-linked immunosorbent assay (ELISA). The premembrane/membrane (prM/M) and envelope (E) proteins of JE virus were expressed in HeLa cells infected with a recombinant vaccinia virus that encodes the JE virus prM and E genes and were released from cells in a particulate form. The particulate antigens were partially purified from culture fluid from the infected cells by precipitation of particles with polyethylene glycol and then dissociated from the particles with 0.1% Triton X-100. This antigen preparation was used to evaluate one preimmune and two postvaccination sera from 20 volunteers given three inoculations of the commercial JE vaccine (Biken vaccine) by a conventional ELISA. The results from this assay correlated with neutralization data. The results of an IgM capture ELISA carried out with the recombinant antigen also correlated with the results of an existing IgM capture ELISA performed with JE virus-infected mouse brain, when tested with 29 serum and 13 cerebrospinal fluid samples from JE patients. These results indicated that recombinant JE virus antigens are useful for ELISA as an antigenically equivalent, highly productive, and safe alternative to authentic JE virus antigens.

KEY WORDS: capture ELISA, IgM, IgG, recombinant vaccinia virus

INTRODUCTION

Existing tests for serodiagnosis of flavivirus infections involve complement fixation, hemagglutination (HA) inhibition, neutralization (NEUT), and enzyme-linked immunosorbent assay (ELISA) tests [Beaty et al., 1989]. Usually, the antigens used for these tests are prepared from mouse brain or cultured cells infected with flaviviruses, raising safety concerns especially for viruses such as Japanese encephalitis (JE) virus, which should be propagated using strict biosafety practices. For the ELISA, crude viral antigens often produce nonspecific reactions with human sera so that purification of antigens from the virus-infected preparations is often needed. This procedure can be both laborious and unsafe. Recently, production of viral proteins without replication of the homologous virus has been made possible by recombinant technology. The ability to produce recombinant flavivirus antigens raises the possibility that these antigens could be used in place of existing authentic antigens, eliminating biosafety problems.

We constructed recombinant vaccinia viruses encoding the JE virus premembrane (prM) and envelope (E) proteins, which are able to produce the prM and E proteins equivalent to the authentic proteins in infected cells. Cells infected with these recombinant vaccinia viruses release these proteins in the form of extracellular particles (EPs) from infected cells [Mason et al., 1991; Konishi et al. 1991], which differ from authentic virus by the absence of the C protein and the viral RNA [Konishi et al., 1992]. The yield of the E protein is approximately 5–10 μg/ml of culture fluid of infected cells. Based on antigenicity, safety, and yield in cell culture, the EP antigen is an excellent candidate as an alternative antigen for use in conventional JE serologic tests. This study has evaluated this recombinant antigen in conventional and capture ELISAs for JE diagnosis.

MATERIALS AND METHODS

Antigen Preparation

The procedure for purification of EPs [Konishi et al., 1992] was simplified for partial purification of recombi-

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nant antigens. HeLa cell monolayers grown in 150 cm^2 flasks were infected with a recombinant vaccinia virus encoding prM and E genes of JE virus (vP829) at a multiplicity of infection of 2. After infection, cells were rinsed three times with Eagle's minimal essential medium (MEM) and maintained in MEM containing 0.1% bovine serum albumin (BSA) for 24 hr at 37°C. Culture fluids were harvested, clarified, and then incubated for 2 hr at 4°C with polyethylene glycol 8000 (PEG; Sigma Chemical Co.) and NaCl at final concentrations of 10% and 1.9%, respectively. PEG-precipitated material was collected by centrifugation at 10,000 rpm for 20 min in a Sorvall SS-34 rotor. The pellet was dissolved in a small amount (1/100 volume of culture fluid) of 0.1 M carbonate buffer (pH 9.6) containing 0.1% Triton X-100 (TX100). After clarification, this preparation was used as antigen for ELISAs. The control antigen was prepared by the same procedure from culture fluid of cells infected with vaccinia virus vP410, which does not express JE antigens.

Sera

Serum samples from 20 vaccinees who received three doses of inactivated JE vaccine (Biken vaccine) were supplied by the Walter Reed Army Institute of Research, with NEUT titers supplied by the Yale Arbovirus Research Unit (YARU). These samples included three sera serially collected from each recipient: day 0 (preimmune), day 60, and day 180 after immunization. The recipients had no history of yellow fever (YF) vaccination, and the sera were negative for antibodies to YF virus as determined by ELISA at YARU. Serum and cerebrospinal fluid (CSF) samples from JE patients were supplied by the Department of Virology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Thailand, and the Department of Virology, National Institute of Health, Korea.

Conventional ELISA

The conventional ELISA for detecting IgG antibodies to JE virus was carried out essentially as described previously [Konishi and Yamaoka, 1982]. Microplates (Maxisorp, Nunc; Immulon 4, Dynatech) were sensitized with recombinant and control antigens at a 1:800 dilution overnight at 4°C and blocked with 1% BSA at 37°C for 30 min. This dilution of recombinant antigen provided 30 ng E per well. The plates were then incubated with test sera at a dilution of 1:100 at 37°C for 1 hr, with alkaline phosphatase-conjugated anti-human IgG (γ-chain specific; Tago Inc.) at a dilution of 1:1,000 at 37°C for 1 hr, and with 1 mg/ml *p*-nitrophenyl phosphate at 37°C for 10 min. The diluent for test sera and conjugate was phosphate-buffered saline (PBS) containing 1% BSA, 0.05% Tween 20, and 0.02% sodium azide, and the buffer for the substrate was 10% diethanolamine buffer (pH 9.8) containing 0.01% $MgCl_2$. The difference in absorbance values obtained with recombinant and control antigens was regarded as antibody levels specific for JE antigen.

Capture ELISA

The capture ELISA for detecting IgM antibodies to JE virus was carried out using the method described by Burke et al. [1985] with some modifications. Briefly, microplates were sensitized overnight at 4°C with rabbit anti-human IgM (μ-chain-specific; Cappel Laboratories, Inc.) at a dilution of 1:1,000 and blocked with BSA. Plates were then incubated with test samples at a dilution of 1:100 at 37°C for 1 hr, with recombinant and control antigens at a dilution of 1:500 at 37°C for 1 hr, with horseradish peroxidase-conjugated human anti-flavivirus (IgG from pooled convalescent sera of patients recovering from dengue virus infection) at a dilution of 1:200 at 37°C for 1 hr, and with 1.8 mg/ml *o*-phenylene diamine in the presence of 0.03% H_2O_2 at 37°C for 20 min. Diluent for antigens and conjugate was 20% acetone-extracted normal human serum in PBS containing 0.05% BSA, supplied by AFRIMS. In parallel, the original IgM capture ELISA was carried out using sucrose-acetone extraction of infected and normal mouse brains at a dilution of 1:50 as antigens [Burke et al., 1985]. The difference of absorbance values obtained with recombinant and control antigens was compared with the difference of absorbance values obtained with infected and normal mouse brain antigens.

RESULTS

The amount of recombinant JE virus E protein recovered from the culture fluid of vP829-infected HeLa cells depended on the concentration of BSA contained in the medium. A direct ELISA to compare the amount of E antigen in culture fluid indicated that higher E protein yields were obtained with BSA concentrations of 1% and 0.1% than with 0.01% or less. Optimal recovery of E antigen was obtained with a PEG concentration of 10%. Under these conditions, 25–50% of the total E present in culture fluids was recovered from the precipitated fraction. The PEG-precipitated preparation contains approximately 250 μg of E and 125 μg of BSA per milliliter, as estimated from Coomassie blue staining of samples resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The E protein and BSA were the major proteins contained in the PEG-precipitated preparation. In conventional ELISA, stronger reactions were observed when the EPs were pretreated with TX100. Although the basis for this finding was not investigated, it is possible that washes containing Tween 20 could have dissociated the lipid-containing particles, removing a portion of the antigens. In contrast, antigens dissociated from EPs before sensitization may have attached on the plate more effectively. Dose-response curves of different sera indicated that the E protein at a concentration of 300 ng/ml was suitable for differentiating positive from negative sera; this corresponded to a 1:800 dilution of the PEG-precipitated recombinant antigen preparation.

The conventional ELISA using recombinant antigens was compared with the NEUT test using 60 sera obtained from Biken vaccinees on days 0, 60, and 180

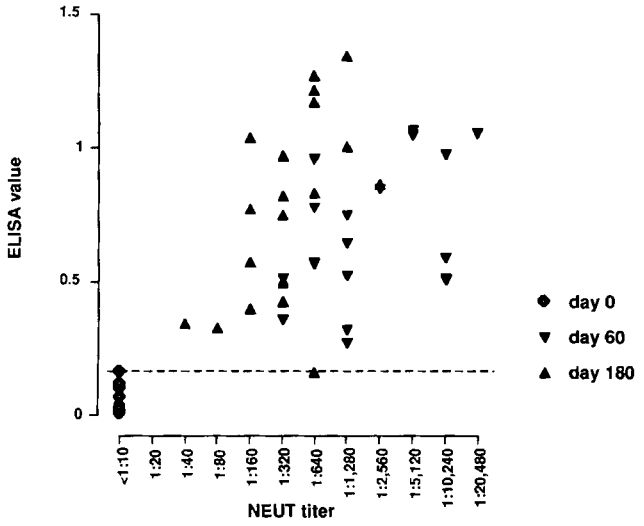

Fig. 1. Comparisons between NEUT and conventional ELISA tests for IgG antibodies to JE virus using 60 samples obtained from vaccinees 0, 60, and 180 days after immunization. A broken line indicates the borderline calculated from absorbance values obtained with 20 samples from day 0 (mean +3 × standard deviation).

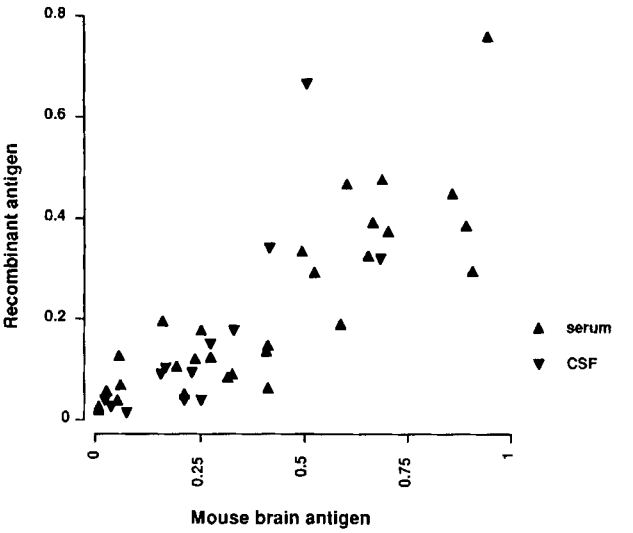

Fig. 2. Comparison between IgM capture ELISAs using recombinant antigens and mouse brain antigens, with 29 serum and 13 CSF samples of JE patients. Ordinate and abscissa indicate absorbance values obtained from ELISAs using the antigens indicated.

after immunization (Fig. 1). A significant correlation was observed between these methods ($r = 0.792$, $P < 0.001$). One of twenty samples from day 180 that were positive for NEUT were lower than the mean +3 × standard deviation (0.159) obtained with 20 preimmune sera that were negative for NEUT. Based on this borderline result, complete qualitative consistency was observed with the 60 days postimmunization samples. The IgM capture ELISA using recombinant antigens correlated significantly with the original IgM capture ELISA using mouse brain antigens, using 29 sera ($r = 0.851$, $P < 0.001$) and 13 CSF samples ($r = 0.778$, $P < 0.001$; Fig. 2).

DISCUSSION

Significant correlations obtained by both conventional and capture ELISA systems demonstrate that recombinant JE virus antigens can replace authentic JE virus antigens in these tests. The correlation between the NEUT test and the conventional ELISA was expected, because the recombinant antigen contains correctly folded forms of E [Konishi and Mason, 1993], and the epitopes reactive with NEUT antibodies are located predominantly on the E protein of flavivirus virions [Heinz, 1986]. Similarly, the results suggest that antigens responsible for antibody reactions in IgM capture ELISA seem to be mainly prM/M and E proteins included in infected mouse brain antigens. Furthermore, as was shown previously, a particulate form of recombinant antigens (EP) obtained from vP829-infected HeLa cells has HA activity similar to that of JE virions in terms of the optimal pH and the inhibition by anti-JE virus antibody [Konishi et al., 1991], indicating that EPs may be also used for the HA test as an alternative antigen.

The applicability of recombinant antigen to these serologic tests is based on the fact that these proteins are produced properly in cells infected with vP829. A previous study suggested that EPs are produced on the endoplasmic reticulum membrane of the cell (in which prM and E are accumulated) in a way similar to particle formation occurring in JE virus-infected cells [Konishi and Mason, 1993]. Comparison in reactivity to nine species of anti-E monoclonal antibody indicated that the E protein produced in vP829-infected cells has antigenic properties similar to those of E produced in JE virus-infected cells. The antigenicity was confirmed by animal experiments in which mice immunized with either vP829 or EPs elicited NEUT antibodies [Konishi et al., 1992].

Minor contamination of vaccinia virus antigens in recombinant antigen preparation seems to affect the results of conventional ELISA with sera containing antivaccinia virus antibody, in that human serum samples showed various antibody levels using control antigens prepared with a parent vaccinia virus (vP410; results not shown); however, none of these reactions inhibited the ability to detect anti-JE virus antibodies. Antigen expression systems that do not produce proteins derived from vectors, such as a continuous expression system in mammalian cells, may have an advantage over vaccinia expression systems. However, IgM capture ELISA almost did not react with control antigens as far as the present 42 samples were concerned, consistent with the principle that capture ELISA systems are able to select specific antigens and to eliminate contaminating antigens. It will be important to apply this technique to serodiagnosis of other flavivirus infections, especially dengue, which is another serious public health problem in tropical areas.

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